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The three leg imaginal discs of *Drosophila*: "Vive la différence"

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ABSTRACT

The imaginal discs of *Drosophila* are the larval primordia for the adult cuticular structures of the adult fly. Fate maps of different discs have been generated that show the localization of prospective adult structures. Even though the three legs differ in their morphology, only the fate map for the T1 (prothoracic) leg disc has been generated. Here we present fate maps for the T2 (meso-) and T3 (metathoracic) leg discs. We show that there are many similarities to the map of the T1 leg disc. However, there are also significant differences in the contributions of each disc to the thorax, in the morphology of joints connecting the legs to the thorax, in bristle patterns, and in the positioning of some sensory organs. We also tested the developmental potential of disc fragments and observed that T2 and T3 leg discs have more limited plasticity and are unable to transdetermine.

The differences in the cuticle patterns between legs are robust and conserved in many species of dipterans. While most previous analyses of imaginal disc development have not distinguished between the different leg discs, we believe that the underlying differences of the three leg discs demonstrated here cannot be ignored when studying leg disc development.

Introduction

Studies of genes involved in axis specification have shown that the same rules apply to all three pairs of legs in *Drosophila*: *en/inv* regulates anterior/posterior fates, whereas dorsal–ventral and proximo-distal axes are under the control of *wg* and *dpp*. This has led many researchers to lump all three leg discs into one group when analyzing the function of genes (Gilbert, 2010). For example, the Gal 4/UAS system (Brand and Perrimon, 1993) has extensively been used to direct gene expression in imaginal discs. Overexpression of a gene has been analyzed indiscriminately in any of the leg discs, assuming it generates the same phenotype. But surprisingly increased *dpp* expression in its normal domain produces a different phenotype in the T1 leg than in the others (Morimura et al., 1996).

Further, it has been known for a long time that the legs differ in their size and shape, as well as in their bristle patterns (Hannah-Alava, 1958). More recent studies on the femur, tibia, and first tarsal segments have demonstrated the role of Hox genes in size regulation and patterning of these segments. *Scr* expression defines T1 leg identity and *Ubx* defines that of the T3 leg. It is postulated that the transverse rows found in T1 and T3 legs have evolved from the more elementary T2 leg pattern (Struhl, 1982; Stern, 2003). Evolutionary biologists have identified Hox gene function in leg discs that regulate the different morphologies in the three leg discs whereas most developmental biologists do not make the distinction between the different leg discs. This has stimulated us to have a closer look at the morphology of the three leg pairs, in particular the size and shape differences of the coxa and trochanter, and the groups of sensilla campaniformia and trichodea. Interestingly, in *Drosophila melanogaster* these sensory fields have only been described for the T1 leg (Hodgkin and Bryant, 1978). Then again, Frantsevich and Gladun (2002) describe sensory fields in 205 insect species (including *Drosophila*), but this study focuses only on T2 legs and only on coxa and trochanter.

We previously generated a fate map of the T1 leg disc and it is often used as a generic fate map for any of the legs. In light of the differences in leg morphology we now have generated fate maps for T2 and T3 leg discs.

We report here that transplantation of entire discs directly into metamorphosing larvae has identified differences in thorax contributions of the three different leg discs. Earlier clonal analysis already has indicated that the T2 leg disc forms the sternopleura of the thorax (Steiner, 1976; Wieschaus and Gehring, 1976), but our transplantation data provides direct evidence for this. Steiner's analysis of early induced clones also has shown that the T2 and T3 leg discs are restricted to hemisegments of either anterior or posterior compartment identity but can produce structures of both wing and leg. This is not the case for the T1 leg where clones induced up to the mid first instar show left–right overlaps. This further indicates a clear difference

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in developmental progression between the thoracic legs (Steiner, 1976).

To generate the fate maps, we mostly use the sensory fields (for example groups of sensilla trichodea, or sensilla campaniformia) since they are easy to identify and show little or no variations in the number and arrangement of the sensilla. Despite the similarity of the fate maps of the three leg types, we find clear differences in the position and number of specific sensillar groups. This has led us to a brief comparison of these sensillar groups in a number of species and we show that these differences are extremely well conserved even though the size of the appendages can vary greatly.

Material and methods

Fly stocks

For all experiments we used the "Sevelen" wild-type stock raised at 25 °C on standard food (cornmeal, molasses, agar, and yeast). We collected eggs from age-matched adults for 15 min after a 1-h pre-collection to minimize over-aged embryos. We dissected discs from wandering larvae 100 h after egg deposition (AED). Discs were handled in Ringer's solution (Schubiger, 1971). To analyze *btd*-expression, we crossed *btd-Gal4* flies (a gift from R. Mann) to flies carrying *UAS-GFP*.

Fate mapping

We fragmented discs using thin tungsten needles (diameter at the tip 10 μm), sharpened electrolytically in a 1 M NaOH solution. Such needles only damaged one cell layer beyond the cut site of the disc (Reinhardt and Bryant, 1981 Poodry, pers. comm.). We are aware that a cut through an imaginal disc cuts through two cell layers: the peripodial layer and the disc proper, however the peripodial layer contributes little to the adult leg. For fate mapping experiments, discs were cut in half to generate four types of fragments: Upper (1/2 Up) and lower (1/2 Lo) halves, and medial (1/2 M) and lateral (1/2 L) halves (Fig. 5). These terms were used by others and us to designate the fragments (Bryant, 1978; Schubiger, 1968; Steiner, 1976), and refer strictly to disc morphology, and not to anterior/posterior compartments or the views of the adult leg. Still, the 1/2 M fragment is mostly anterior compartment and the 1/2 L fragment mostly posterior (Figs. 1 and 9). Bisection of discs into anterior (A) and posterior (P) compartments would be ideal for this study however the compartment boundary is not a straight line and there are no morphological markers in imaginal discs to properly orient the cuts. In contrast, we relied on the shape of the discs (the tip), the disc central point (end knob, EK), the lowest point of the disc, and points such as the nerve to define accurate and reproducible cut positions (Fig. 1).

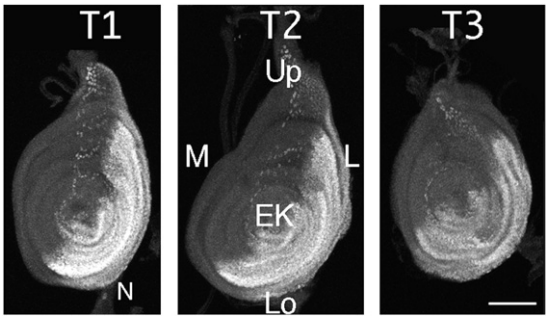


Fig. 1. The three imaginal leg discs. (A) Pro- (T1), meso- (T2) and metathoracic (T3) leg discs labeled with anti-En to indicate the posterior compartment (note that the compartment boundary does not run in a straight line). EK: endknob (primordium of tarsal segments 2–5), L: lateral, Lo: lower, M: medial and Up: upper aspects of the disc. N=nerve. Scale bar=50 μm.

Pro-, meso- and metathoracic adult legs were abbreviated as T1, T2, and T3 legs formed from pro- meso- and metathoracic discs we called T1, T2 and T3 leg discs. The 5 tarsal segments were designated as Ts 1-5. Although the T1 leg disc has already been fate-mapped (Schubiger, 1968), we performed the current analysis with all three disc types because the fragments used here have not been reported on previously. Furthermore, our new observations required minor changes to the original fate map of the T1 leg disc (Fig. 9A and C).

We injected fragments into late wandering larvae (110 h AED), just a few hours before puparium formation, to minimize the window of cell division in the host before they differentiated adult structures. Overall, 60–70% of hosts survived as adults. 1–2 days after adult eclosion, implants were retrieved, incubated in 5 M KOH for 5 min, washed several times in water, and dissected in a drop of Faure's water mounting medium (Ashburner, 1989). Before mounting the cuticle, we further dissected the vesiculated segments to achieve one or two layer(s) of cuticle. This made it easier to identify and analyze the cuticle of specific structures.

For the characterization of cuticular structures we used the nomenclature by Steiner (1976) based on terms used by Zalokar (1947).

In vivo culture

To test the developmental capacity of the T2 and T3 leg disc (Fig. 10), we injected 3/4 L fragments into adult female hosts within 24 h of eclosion, that is, before their ovaries had grown to maturity. This made recovery of discs fragments easier. Survival rate of the adult hosts in most experiments was 90% or higher. Following in vivo culture, the fragments were injected into larval hosts to allow cuticle differentiation, as described above. After analyzing the cuticular structures, we compared the T2 and T3 data with the previously published results for T1 (Abbottt et al., 1981).

Imaging

Cuticle images were collected as *z*-series stacks on a Leica SP5 II confocal microscope using 40× or 63× oil immersion objectives. In situ legs (Figs. 2A and 3A) were mounted on a slide that included a layer of double-stick tape between the slide and coverslip to maintain depth between the two cuticle layers, and imaged with the Leica tile scan function. To separately display the anterior and posterior structures, the stacks were split in half and projected as two separate images using Image J 1.44, then stylized using the "Find Edges" filter. In all other cuticle images, specific structures from a *z*-series were cut and pasted into a single image to optimize their focus.

Immunohistochemistry

Leg discs were dissected in PBS, fixed for 20 min in 4% formaldehyde and rinsed several times in PBS+ 0.2% Triton-X. We used the following antibodies: mouse anti-En (4D9, Developmental Studies Hybridoma Bank, 1:50), guinea pig anti-Hth (R. Mann, 1:2000) and mouse anti-GFP (Invitrogen, 1:500).

Results

In situ description of the different leg segments

Before generating a fate map, let us describe the morphological differences between the three pairs of leg discs. We measured the

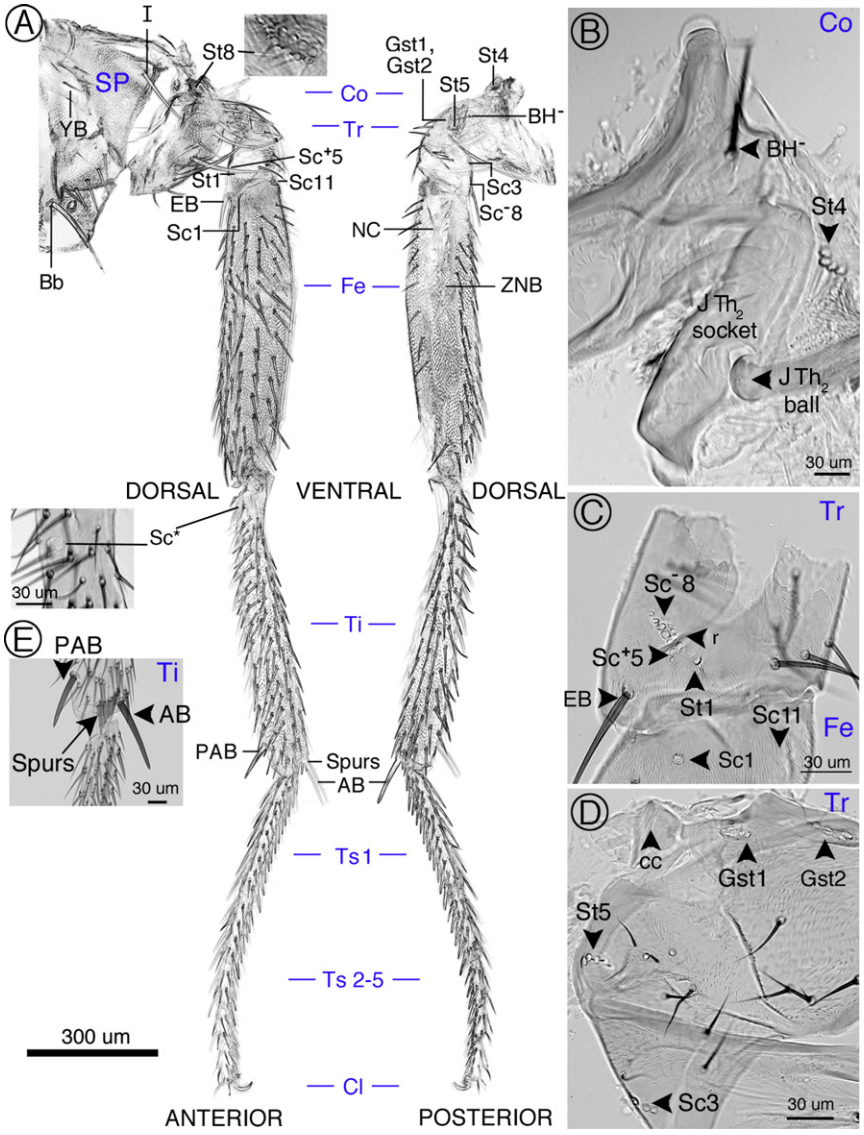


Fig. 2. The structures of the T2 leg, illustrated with in situ leg cuticle (A, E) and cuticle from transplants (B, C, D), where a better layout of the markers is often found. (A) Anterior and posterior views of a ventral-dorsally-split T2 leg including sternopleura (SP), the isolated single bristle (I), the three yellow bristles (YB), and the group of bristles including one or two very big ones (Bb). In the coxa (Co), we find 8 sensilla trichodea grouped into two rows (St8). (B) The coxa has an isolated bristle (BH-), a row of 4 Sts (St4), a joint including a socket (J Th2) and a ball (J Th2) in the SP. (C and D) The trochanter (Tr) has many good markers: 5 Sensilla campaniformia in a hairy background ($Sc^{+}5$), 8 Scs on naked background ($Sc^{-}8$), a cuticular ridge (r) separating the two markers, a single sensillum trichodeum (St1), and the edge bristle (EB) (C). Other trochanter markers include three Scs (Sc3), the row of 5 Sts (St5), a round cuticle circle (cc), and the two groups of sensilla trichodea (Gst1, Gst2) (D). In (C), a single Sc (Sc1) and a group of 11 Scs (Sc11) in the femur (Fe) are seen. In the femur the posterior side has a zone of no bristles (ZNB), and naked cuticle (NC) towards the trochanter (A). At the proximal part of the tibia (Ti) the site of 4 single Scs * are indicated (A), also known as the tibial sense organ (Hodgkin and Bryant, 1978). At the distal end of the Ti and on the dorsal side, the bractless preapical bristle (PAB) is found. (E) The spur bristles (Spurs) surround the apical bristle (AB). The tarsus has 5 segments (Ts, 1–5) and a claw organ (Cl) at the distal end (A). Blue letters indicate leg segments.

projected area of the posterior compartment in discs labeled with anti-En. Although the discs were similar in size and shape, the posterior compartment of T1 and T2 leg discs in early wandering larvae was generally larger than in the T3 leg discs ($p=0.01$, Mann-Whitney rank sum test). To quantify subtle differences in shape, we calculated a length to width ratio (measuring the length of a straight line along the anterior/posterior boundary), and found that T1 leg discs were significantly longer, whereas the T3 leg discs were rounder ($p<0.01$, $n=18$, 9, Tukey's test). The ratio for T2 leg discs from early wandering larvae was larger than the ratio in T3 legs, but as the discs matured, T2 and T3 leg discs had similar ratios ($p=0.96$, $n=12$, 14, Tukey's test). We did not notice any consistent differences in the shape of the posterior compartment as evidenced by En expression between the different leg discs (Fig. 1).

Comparing adult legs, the coxa of T1 leg was clearly much larger than in the T2 leg, whereas the thorax of T2 legs was much more substantial. The coxa and thorax of T3 legs were the smallest (Figs. 2 and 3). Thus we asked if the shape and size of the coxal anlage is different in the three leg discs. We defined the area of the coxa anlage as the region of the disc where Hth (Homothorax) and Btd (buttonhead) expression overlapped (Estella and Mann, 2010, Fig. S1). In mid-third instar leg discs, however, we were unable to detect a clear difference between the different leg discs (Fig. S1).

Previous careful descriptions of the bristles of the fly leg were limited to the femur (Stern, 2003), the tibia, and tarsus (Hannah-Alava 1958; Held, 2002; Tokunaga, 1962). Furthermore, Steiner (1976) used clonal analysis to group adult leg bristles of all three leg types to the anterior or posterior compartments. However,

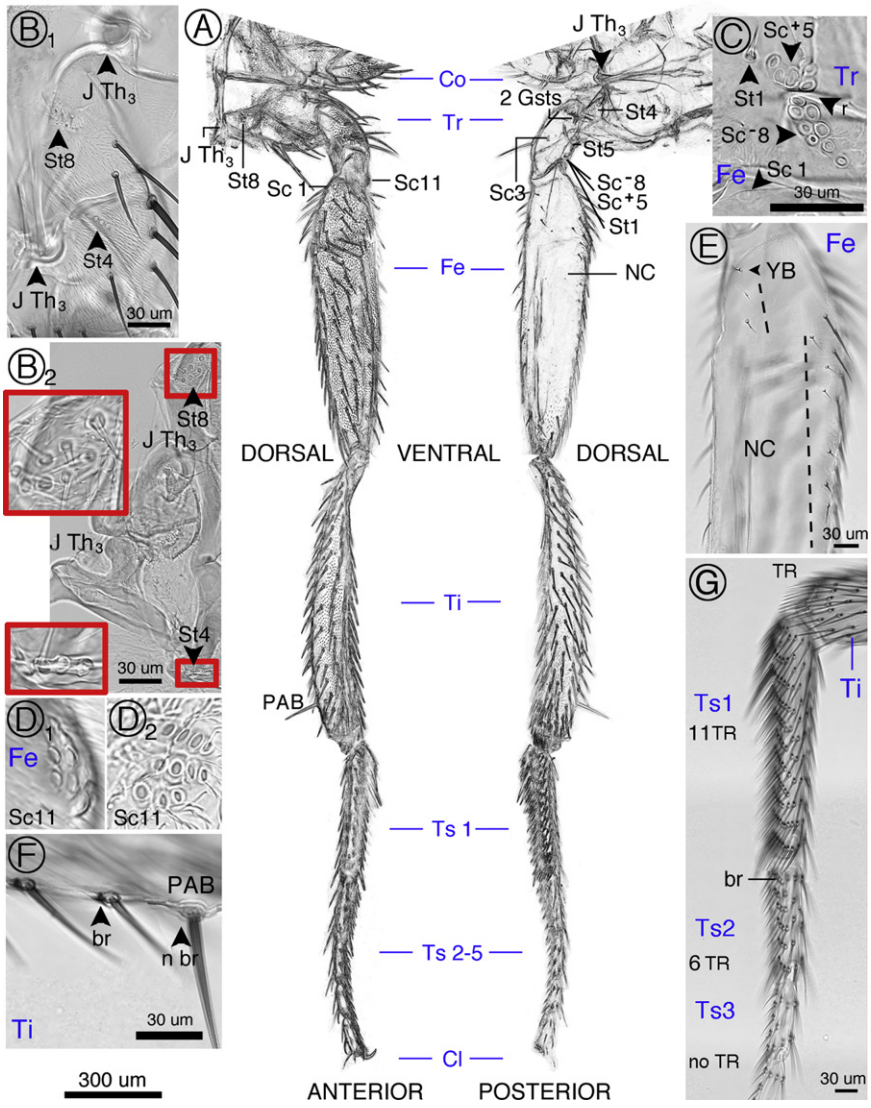


Fig. 3. The structures of the T3 leg, illustrated with in situ leg cuticle (A, B1, D1, E, F, G) and cuticle from transplants (B2, C, D2). (A) Anterior and posterior views of a ventral-dorsally split T3 leg. For abbreviations, see Fig. 2. (B1) The T3 coxa (Co) has two thoracic joints (J Th_3). (B2) shows the same region differentiated from a transplanted whole disc. We observe two joints, one near St8, the other near St4 (red boxes). In transplants, the two rows of the St8 are often not well aligned (B2), as was also seen with St4 (not shown). In (C), we introduce the trochanter (Tr) markers (St1, $Sc^{+}5$, $Sc^{-}8$). Unlike T2, EB is not found between $Sc^{+}5$ and $Sc^{-}8$, though a cuticular ridge (r) is. On the dorsal and proximal aspects of the femur (Fe) we find Sc1. Again, proximally on the ventral side we find a group of Sc11 (arranged in 3 rows of 4, 4 and 3 Scs, D2). In T3 legs this structure is difficult to resolve because the cuticle has a domed outgrowth (D1). The arrangement of the 11 sensilla is easier to analyze in transplants (D2). The posterior half of the femur (Fe) has a patch of naked cuticle (NC) bordered by two incomplete rows of small, thin, yellow bristles (YB, dashed lines in E). Distal and anterior in the tibia (Ti) we observe the PAB (A, F). This bristle has no bract (n br) and is prominent because it stands out at a different angle (F). Ti and Ts1 and 2 have bracted transverse row bristles (TR) (G). Blue letters indicate leg segments.

these papers neglected other sensory organs and the joints, the sites of articulation. Legs have extrinsic dicondylic joints with a ball and a socket (Snodgrass, 1927). Since these non-bristle structures were previously described for the T1 leg (Schubiger, 1968; reviewed in Bryant, 1978), we felt a need to carefully describe them in T2 and T3 legs.

When describing the adult cuticular patterns, it is important to distinguished hairs from bristles. A hair (trichome) is an outgrowth from a single epidermal cell, and bristles are multicellular sensory organs (Miller, 1950; Bryant, 1978). Bristles are also known as sensilla chaetica or sensilla trichodea (Miller, 1950). However, we reserve the term sensilla trichodea (St) specifically for very tiny, pale structures. Their distinct morphologies are easy to identify (see for example, the group St8 in the coxa, Figs. 2–5) and their number, unlike the number of bristles, is rather constant. Thus Sts are ideal structures that have been useful for generating detailed fate maps of wing, leg, antenna, and genital discs (reviewed by Bryant, 1978 and for SEM of cuticular structures see Hodgkin and Bryant, 1978). In addition, adult legs have sensilla campaniformia (Sc, Miller 1950). These are domed pale structures (e.g., Fig. 2C), and also represent important fate map markers.

The morphology and cuticular structures of T2 and T3 legs are presented in Figs. 2 and 3. Each leg is shown with an anterior and posterior view based on Steiner's (1976) work. Overall the three legs look very similar in that they all have five segments: coxa (Co), trochanter (Tr), femur (Fe), tibia (Ti), and the 5 tarsal segments (Ts). But there are also significant morphological differences between them. We describe these structures in proximal–distal order:

(1) Thorax: Previously, the contribution of the thorax from the different leg discs was indirectly determined by clonal analysis (Stern, 1963; Bryant and Schneiderman, 1969; Steiner,

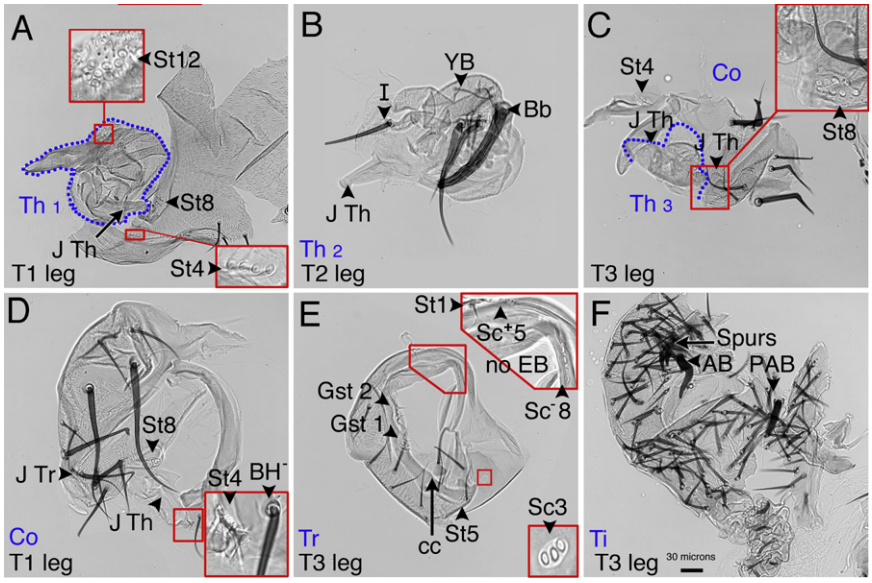


Fig. 4. Leg segments differentiated from disc transplantation controls. (A–F). Thorax contributions from T1, T2, and T3 leg discs (blue dotted line in A and C). (A) The T1 leg disc formed propleura, sternum, episternum 1, the cervical membrane with 12 Sts (red inset), and the ball of joint (J Th) to the coxa. (B) The T2 leg disc formed the sternopleura with the isolated bristle (I), 3–4 yellow bristles (YB), the group of 6–9 bristles including one or two very big bristle(s) (Bb), and the joint (J Th, ball) to the Coxa. (C) The T3 leg disc formed hypopleura and episternum III. In the coxa the St8 can have 9 Sts (red inset). (D) Illustrates a coxa from a T2 leg control with a group of St8, St4, and an isolated bristle on naked ground (BH^-, red inset), the socket of the joint to Th2 (J Th) and the joint to the trochanter (J Tr). In (E), the trochanter formed by a transplanted T3 leg disc. Note that the transplanted T3 leg disc does not differentiate an EB (inset in E). (F) shows tibia bristle patterns differentiated from a transplanted T2 leg disc. While AB, spurs, and PAB can be identified, an arrangement into the different proximal–distal rows cannot be made. Enlarged insets in (A), (C), (D), and (E) are indicated by red lines. For abbreviations see Figs. 2 and 3.

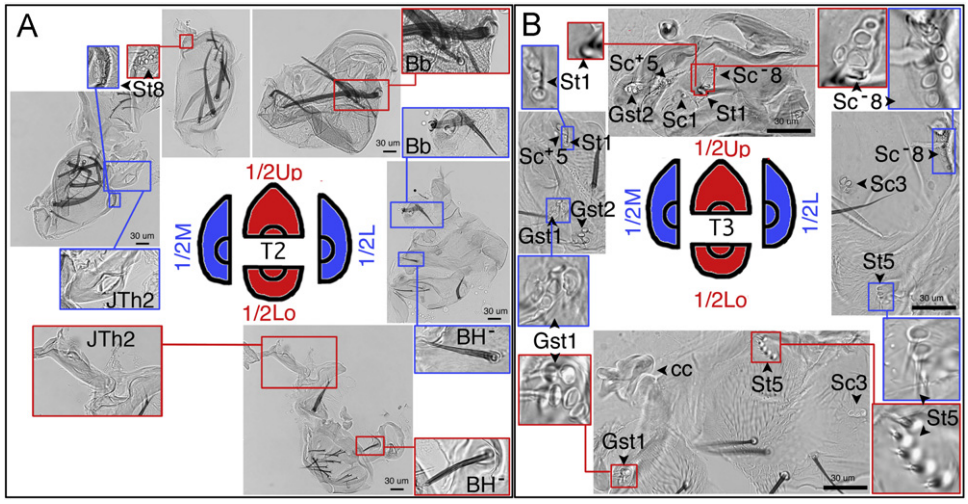


Fig. 5. Method to map cuticular structures to position in the leg disc. Red = upper and lower (1/2 Up and 1/2 Lo) halves; blue = medial and lateral (1/2 M and 1/2 L) halves. (A) The four corners show the thorax and coxa markers that most frequently differentiated from two adjacent T2 disc fragments. For example St8 most frequently differentiated from the 1/2 Up and 1/2 M fragments (also see Fig. 6). (B) The four corners show the trochanter markers that most frequently differentiated from the two adjacent T3 disc fragments (also see Fig. 6). Magnified insets are indicated by red and blue lines. For abbreviations see Figs. 2 and 3.

1976), or described here directly by transplanting whole leg discs into larvae (see below).

The T1 leg disc produces propleura, sternum and, epimerum I (Ferris, 1950; Steiner, 1976; Zalokar, 1947; Schubiger, 1968). These structures have no bristles, but show characteristic hair patterns. The T1 leg disc also produces 12 sensilla trichodea (St12) in the cervical membrane and the ball of a joint (Fig. 4A; J Th), the dorsal articulation with the coxa that was previously mapped (Ferris, 1950; Schubiger, 1968; reviewed in Bryant, 1978). These structures are grouped and called Th1. Hypopleura and sternopleura (Fig. 4B) arise from the T2 leg disc. In the dorsal-posterior aspect there are 5–8 bristles, 1–3 of them is/are very large (Bb). An isolated bristle (I) is located in the ventral aspect of Sp. Lastly, between two and four yellow bristles (YB, Figs. 2A and 4B; Steiner, 1976; Bryant, 1978; Held, 2002) are located anteriorly. We refer to these structures as Th2 (Fig. 4B).

The T3 leg disc produces part of the hypopleura and the episternum 3 with no sensory organs (Steiner, 1976; Bryant, 1978). These structures are referred to as Th3 (Fig. 4C). Th2 and Th3 have ventral joints (articulations) with the coxa (Ferris, 1950) that are difficult to see (Zalokar, 1947).

(2) Coxa: The coxae of the three legs are very similar, but also have some differences. The T1 coxa is at least twice as big as in the T2 and T3 leg. All have cylindrical shapes with longer sides towards the front of the animal (Ferris, 1950; Steiner, 1976).

The anterior half of the front of the coxa is covered with hairs and bristles. The number of bristles differs in that the T1 leg has the most and T3 the fewest (Table S1). This area of the coxa also carries a group of 8 sensilla trichodea (St8) arranged in two rows (Figs. 2A, 3B1, B2, and 4C, D). We found one exceptional coxa from a T2 leg with 9 instead of 8 sensilla trichodea. In the joint to the thorax of T1 legs, two rows of sensilla trichodea are formed (St3 and St4; Schubiger, 1968; Bryant, 1978). On the hind side of the coxa of T1 and T2 legs, a single bristle can be identified (occasionally two) (BH^-, Figs. 2B and 4D) that is never present in T3 legs (Steiner, 1976 and Fig. 3 A). Nearby in all three legs, a row of four sensilla trichodea (St4) is observed (Figs. 2B and 3 B1, B2). Again we found one exception in a T2 leg that had 5 St instead of 4 (St4). The joint between coxa and thorax differs between T1, T2, and T3: T1 and T2 legs have one joint (Schubiger, 1968; reviewed in Bryant, 1978) but in T1 the joint is located dorsally, whereas in T2 the joint is found ventrally (Fig. 2B). The T3 leg has two joints between thorax ($J\,Th_3$) and the coxa. One is near St4, the other near St8 (Fig. 3 B1, B2).

(3) Trochanter: Again this segment has a shape of an obliquely cut cylinder but differs from the coxa in that the hind side is longer. The trochanter of the T2 leg has more bristles than the same segment of the T1 and T3 leg (Table S1). The T3 leg does not have the isolated bristle (edge bristle EB; compare Fig. 2C with 3C; Steiner, 1976). In all three leg types, a group of sensilla campaniformia in this region can be subdivided into a group of 8 (Sc^-8; we observed one exception with 7 in T2 leg), and a group of 5 (Sc^+5, varying between 4 and 7 sensilla). A cuticular ridge can be observed between these two groups of sensilla in all three pairs of legs (r, in Figs. 2C and 3C). Also in the distal region of this leg segment, we find a single sensillum trichodeum (St1, Figs. 2C, 3C) in all three legs. Proximal in this segment and near the joint to the coxa, two groups of sensilla trichodea (Gst1 and 2) are formed in all three legs and vary between 5 and 7 sensilla (Fig. 2D). We could only precisely count the number of sensilla in 60% of the cases due to folding of the cuticle. Thus we do not know whether the variation is real. In the analyzable material no variation is seen in Sc3 and in St5 (Fig. 2D). In addition, we identified a specific circular cuticular (cc) structure between St5 and one of the two Gsts. We used this structure to designate Gst1 as the one closest to it (Figs. 2D, 4E). We also observed slight differences in the hair pattern of the three legs. In leg T3 the marker Sc^+5 is not surrounded by hairs as in leg T1 and T2 (Fig. 9C).

(4) Femur: The role of *Ubx* in controlling morphological differences in this segment in the three legs has been well-studied (Stern, 1998; Rozowski and Akam, 2002). As already described by Stern's group, bristles and trichomes differ between the three leg pairs. The femur of T1 leg carries many bristles arranged in proximal–distal rows (Steiner, 1976; Rozowski, 2002). Steiner defined four longitudinal bristle rows by clonal analysis in the anterior compartment, but was unable to do so in the posterior compartment. In addition, it has been observed that proximal segments with increased diameter have more bristle rows but the number of rows is difficult to define because bristle arrangement is more irregular (Hollingsworth, 1964). In the posterior compartment of T2 legs many of the bristles are missing, including the very large bristles (zone of no bristles, ZNB; Fig. 2; Hollingsworth, 1964; Steiner, 1976; Stern, 1998, 2003). In T3 legs this cuticular region is unique, neither bristles nor hairs are found (NC, Fig. 3E; Steiner, 1976; Stern, 2003). This naked region is bordered by two incomplete rows of short, yellow bristles running proximo-distally (YB; Fig. 3E). At the proximal end of this segment, toward the backside, all three legs display 11 sensilla campaniformia (Sc11) arranged in 3 rows with 4, 4, and 3 sensilla (Fig. 3D; one case had 4, 4, 4 arrangement). In transplantation experiments this arrangement is more variable. The front sides of the femora show a single sensillum campaniformum (Sc1; Figs. 2C and 3C).

(5) Tibia: Four Scs and four Sts are observed in the proximal part of this leg segment and in all three pairs of legs (Fig. 2; Held, 2002; Schubiger, 1968; Hoikkala and Moro, 2000). In terms of bristle patterning, this segment differs most between the three different leg pairs. The anterior side of the T1 tibia has 5–7 easily identifiable transverse rows (TRs), with bristles that are thin, straight, more yellow, and without bracts (black triangles above the sockets; Schubiger, 1968; Held, 2002; Bryant, 1978; Hannah-Alava, 1958). Hollingsworth (1964) and Held et al. (1986) distinguished ten longitudinal rows of mechanosensory bristles around the circumference. Two longer and thicker bristles, one of them in the analogous position of the apical bristle (AB) in the leg T2 (Fig. 2E), have been placed in the anterior compartment by clonal analysis (Steiner, 1976). We define preapical bristles (PAB) for all three legs (T1, T2, and T3) because of their morphological and positional similarities (Hannah-Alava, 1958).
The tibia of leg T2 is thought to be the most primitive because it has no TRs (Held, 2002; Hollingsworth, 1964). In this leg there are two distinguishable bristles without bracts: The PAB and AB (Figs. 2E and 4F). They are both located in the anterior compartment: one on the dorsal, the other on the ventral side (Hannah-Alava, 1958). PAB is part of row 5, and AB belongs to row 8 (Steiner, 1976; Figs. 2E and 4F). The AB bristle is surrounded by four or five short, thick, and bracted bristles (spurs) placed in the anterior compartment (Figs. 2E and 4F; Steiner, 1976).
The tibia of leg T3 has one single TR in the posterior compartment (Hannah-Alava, 1958) between and including rows 2 and 3 (Steiner, 1976).

(6) First tarsal segment (Ts 1, or basitarsus):
The three pairs of legs do not differ in the arrangement of bristles into eight longitudinal rows; row one being at the ventral position (originally described by Hannah-Alava, 1958).
T1 leg: TRs are located in the anterior compartment and placed between proximal–distal rows 7 and 8 (anterior, ventral, Steiner, 1976; Hannah-Alava, 1958; Held, 2002). Here big differences between males and females are observed. Males have 5–6 TRs of bracted bristles, whereas females have two additional TRs (Hannah-Alava, 1958; Bryant, 1978; Held, 2002). In males, these two rows are replaced by the sex comb and by one central bristle (Hannah-Alava, 1958; Bryant, 1978; Tokunaga, 1962; Held, 2002).
T2 leg has no TRs but has two longitudinal (proximal–distal) rows of thick and shorter bristles on the ventral side (rows 1 and 8; Hannah-Alava, 1958; Held, 2002). Again Ts 1 of leg 2 has no TRs and is thought to be the most primitive (Hollingsworth, 1964; Davis et al., 2007).
T3 leg has 11 TRs again located in the posterior compartment that occupy the region between row 1 and 3 (Steiner, 1976; Hannah-Alava, 1958; Held, 2002; Stern, 2003).

(7) Tarsal segments (Ts) 2–5:
Only T3 leg has TRs. They are easy to see in Ts 2 but are difficult to identify in Ts 3 (Fig. 3G). The claw organ is at the distal end of Ts 5.

Patterning of transplanted leg discs compared to normal adult legs

Before performing fate mapping experiments, we tested whether the method itself (transplantation of imaginal discs into larval hosts where they differentiate cuticular structures in the

metamorphosing host) affected the normal patterning process. Therefore we compared the normal, in situ patterns of T2 and T3 legs with the structures from transplanted whole discs. Such a comparison was previously performed with T1 leg discs, and no qualitative difference was observed, but bristle numbers of all leg segments were reduced and had larger standard deviations (Schubiger, 1968; Tab. S 1). Bigger variations were also observed in the number of sensilla in the sensory fields.

During normal development, leg discs become concentrically folded, with the most distal primordia in the center and the proximal primordia at the periphery. This is reflected in the concentric-ring expression of proximal–distal segmentation genes. *Homothorax (hth)*, for example, can be used to follow proximal segments, and *Distal-less (Dll)* for distal segments (for review, see Estella et al., 2012). During metamorphosis the segments telescope out and form the elongated appendages (Bodenstein, 1950; Fristrom and Fristrom, 1975, 1993).

In whole disc transplantation experiments, one major difference is that discs fail to evert. The cuticle appears as rings, in contrast to the more cylindrical shape of normal adult segments (compare Fig. 4 with Figs. 2A and 3A). This same phenotype is found in pharate adults of mutants where legs fail to evert. The ring-like arrangement of the segments is especially clear after careful cuticle dissection (Fig. 4D and E).

After whole disc transplantation, we found all the patterns and markers in the proper arrangement and position. However, while transplantation did not qualitatively alter the pattern, we note that transplantation did reduce bristle numbers. For example, transplanted T2 leg discs differentiated all bristle types of the sternopleura, (I, Bb, yB), but had only 11.7 ± 2.3 bristles compared with 13.5 ± 1.7 bristles of in situ legs. Such a reduction in bristle number has been observed with all leg and other imaginal discs, and is likely attributed to stress from transplantation rather than to increased cell death (Table S1).

From previous work, the contribution of leg discs to the thorax has been difficult to determine because the thorax in Dipterans is fused into a solid box (Ferris, 1950). Still, results from clonal analysis indicate that discs make a significant contribution to the thorax (Steiner, 1976; Wieschaus and Gehring, 1976). Moreover, Zalokar (1943) ablated T2 leg discs in the 3rd instar and found abnormalities in sternopleura and hypopleura. However, the results were variable and therefore had limited value in generating detailed fate maps (Zalokar, 1943). Here, we transplanted whole discs into metamorphosing larval hosts and subsequently analyzed the differentiated structures. Transplanted T2 leg discs clearly differentiated the YB, I, and Bb of the sternopleura (Fig. 4B), and the ball and socket of the joint between coxa and thorax (JTh_2; Fig. 2B). However, identification of joints in such transplantation experiments was difficult particularly in leg T3. Although we sometimes observed two thoracic joints in T3 (JTh_3; Figs. 3B1, B2 and 4C; Zalokar, 1947), we chose to score only the one joint with the long ball and the socket near St4 of the coxa because this joint was easier to recognize (Figs. 3B1, B2 and 4C).

After whole disc transplantation, the proximal–distal bristle rows in the tibia and Ts 1 could not be identified as such (Hannah-Alava, 1958; Steiner, 1976; Held, 2002). However in most cases AB bristles and PAB were identified (75%; Fig. 4F). In addition the morphology of tibial and tarsal structures was in some cases poor (except TRs and claw organ). In transplants, poor cuticular morphology occurs because the distal primordia involute instead of everting, and this in turn might hinder proper differentiation. In transplants we also observed that bristle numbers in coxa, trochanter and femur were reduced by 5–10%.

As mentioned earlier, some of the markers showed some variations in sensilla number. Even in situ, the trochanter marker $Sc^{+}5$ varied between 4 and 6 sensilla and in disc transplantation controls between 4 and 7, a non-significant difference. Occasionally larger deviations were seen, and in one case a transplanted T3 control had 18 sensilla trichodea instead of 8 in the coxa. In this case we believe that handling of the disc caused damage and overgrowth before differentiation. Such extreme variation occurs in less than 10% of transplants and was never observed in normal development.

The trochanter marker $Sc^{-}8$ varies between 7 and 8 sensilla campaniformia in situ but varied between 7 and 10 in disc transplantation controls. In addition, the sensilla were sometimes spread further apart and the arrangement into rows could be disturbed (St8 of the coxa, Fig. 3B1, B2 and data not shown). Almost no difference in sensilla number was found for St4 (Coxa), St1 (Trochanter) and Sc1 (Femur).

fragment	leg	n	Coxa					
			St8 % (normalized)	J Th* % (normalized)	St3 % (normalized)	St4 % (normalized)	BH⁻ % (normalized)	J Tr % (normalized)
1/2 Up	I st	15	87 (100)	100 (100)	100 (100)	100 (100)	0 (0)	0 (0)
	II nd	27	100 (71)	4 (5)	NF	0 (0)	0 (0)	41 (35)
	III rd	26	100 (89)	8 (10)	NF	4 (4)	NF	15 (25)
1/2 Lo	I st	9	0 (0)	0 (0)	0 (0)	0 (0)	56 (100)	100 (100)
	II nd	19	41 (29)	84 (95)	NF	95 (100)	89 (100)	76 (65)
	III rd	25	12 (11)	76 (90)	NF	96 (96)	NF	44 (75)
1/2 M	I st	17	100 (100)	29 (31)	12 (23)	12 (11)	41 (59)	100 (100)
	II nd	26	100 (100)	81 (100)	NF	38 (38)	19 (23)	92 (100)
	III rd	27	96 (100)	59 (91)	NF	22 (28)	NF	100 (100)
1/2 L	I st	17	0 (0)	65 (69)	41 (77)	100 (89)	29 (41)	0 (0)
	II nd	16	0 (0)	0 (0)	NF	63 (62)	63 (77)	0 (0)
	III rd	18	0 (0)	6 (9)	NF	56 (72)	NF	0 (0)

Fig. 6. Frequencies (%) of coxa markers (St8, J Th * [indicates ball of joint], St3, St4, BH^{-}, J Tr socket) differentiated from four different halves (1/2 Up, 1/2 Lo, 1/2 M, 1/2 L) of the T1, T2 and T3 leg discs. *n*=number of cases; the normalized frequency (in parentheses) reflects the ratio of a structure scored in one half compared to its complementary pair (1/2 Up+1/2 Lo=100% or 1/2 M+1/2 L=100%). NF=not found. For abbreviations see Figs. 2 and 3.

It should be noted that even when a marker was clearly identifiable, we could only accurately count the sensilla in about 60% of preparations, mostly due to suboptimal cuticle mounting.

Fate map comparison of T1, T2, and T3 leg discs

The fate map we previously published for the male T1 leg discs has been used in studies and in textbooks to represent all three different legs (Fig. 9A; Schubiger, 1968). Here we have tested whether such a generalization is justified and to which degree a generic leg fate map is possible. For our fate mapping experiments of T1, T2, and T3 leg discs, two cuts were used (Fig. 5). Discs were either cut "horizontally" to produce an upper and lower half (1/2 Up, 1/2 Lo), or longitudinally to produce a medial and lateral half (1/2 M, 1/2 L). The cuticular structures were scored after fragments had gone through metamorphosis in larval hosts (Fig. 5A and B). For each cut we analyzed between 9 and 27 cases (Figs. 6–8). We note that because leg discs are patterned in concentric rings, the innermost, and therefore smallest, distal segments are more prone to cutting variation and fate-mapping inaccuracies.

As mentioned above we were not always able to identify a particular marker in all cases even though each marker must map to either one of the complimentary fragments. Thus, we recalculated the frequencies of the marker found by designating the combined observed cases from the two complementary fragments as 100%. The normalized frequency is given in parenthesis (Figs. 6–8), and has been used to construct the fate maps (Fig. 9B and C). This calculation is also important for markers that have many Sts or Scs (like $Sc^{-}8$) because complementary pairs can also produce this marker in both fragments.

Steiner (1976) previously used clonal analysis to map the anterior–posterior compartment boundary in all three adult legs. We have extrapolated from his data to include the same compartment boundary in the new disc fate maps (Fig. 9C).

Revision of the original fate map of the T1 leg disc

The data presented here for the T1 leg discs require some minor changes from the published fate map (Fig. 9A; Schubiger, 1968). We have placed Gst1 and Gst2 of the trochanter and Sc11 of the femur more towards the medial side of the disc because Gst1 was found in all 1/2 Lo and 1/2 M fragments (Figs. 7–9A and 9B [violet sector] and 9C [T1]). We have now mapped Gst2 to the upper medial quadrant of the disc because it was only found in 1/2 M and 1/2 Up fragments (Figs. 7 and 9B [orange sector]). Sc11 of the femur was formed by all 1/2 Lo and with about equal

fragment	leg	n	Trochanter									
			St1 % (normalized)	Sc+5 % (normalized)	EB % (normalized)	$Sc^{-}8$ % (normalized)	$Sc^{-}8$ # (normalized %)	Sc3 % (normalized)	St5 % (normalized)	cc % (normalized)	Gst1 % (normalized)	Gst2 % (normalized)
1/2 Up	T1	15	73 (100)	80 (100)	93 (100)	93 (62)	4.8 ± 1.4 (54)	0 (0)	0 (0)	0 (0)	7 (7)	87 (100)
	T2	27	85 (100)	100 (100)	81 (100)	96 (86)	5.9 ± 2.0 (87)	0 (0)	0 (0)	0 (0)	0 (0)	100 (83)
	T3	26	77 (100)	100 (100)	NF	88 (81)	5.4 ± 2.4 (87)	0 (0)	0 (0)	27 (33)	38 (40)	96 (100)
1/2 Lo	T1	9	0 (0)	0 (0)	0 (0)	56 (38)	4.1 ± 0.4 (46)	100 (100)	100 (100)	100 (100)	100 (93)	0 (0)
	T2	19	0 (0)	0 (0)	0 (0)	16 (14)	0.9 ± 1.6 (13)	100 (100)	100 (100)	100 (100)	95 (100)	21 (17)
	T3	25	0 (0)	0 (0)	NF	20 (19)	0.8 ± 1.6 (13)	100 (100)	100 (100)	56 (67)	56 (60)	0 (0)
1/2 M	T1	17	88 (100)	100 (100)	0 (0)	0 (0)	0.0 ± 0.0 (0)	6 (6)	29 (45)	88 (100)	100 (100)	100 (100)
	T2	26	73 (100)	100 (100)	0 (0)	0 (0)	0.0 ± 0.0 (0)	0 (0)	23 (21)	77 (93)	100 (100)	100 (100)
	T3	27	35 (100)	89 (100)	NF	0 (0)	0.0 ± 0.0 (0)	0 (0)	22 (22)	56 (100)	96 (100)	100 (100)
1/2 L	T1	17	0 (0)	0 (0)	65 (100)	94 (100)	7.6 ± 0.7 (100)	94 (94)	35 (55)	0 (0)	0 (0)	0 (0)
	T2	16	0 (0)	0 (0)	56 (100)	100 (100)	6.5 ± 1.8 (100)	100 (100)	88 (79)	6 (7)	0 (0)	0 (0)
	T3	18	0 (0)	0 (0)	NF	100 (100)	7.3 ± 1.1 (100)	78 (100)	78 (78)	0 (0)	0 (0)	0 (0)

Fig. 7. Frequencies (%) of trochanter markers (St1, $Sc^{+}5$, EB, $Sc^{-}8$, Sc3, St5, cc, Gst1, Gst2) differentiated from the four different halves of the T1, T2, and T3 leg discs. *n*=number of cases. $Sc^{-}8$ #: mean number of Sc produced. NF: not found. Parentheses: as in Fig. 6. For abbreviations see Figs. 2 and 3.

fragment	leg	n	Femur			Tibia			Ts 1	Ts 2-5
			Sc1 % (normalized)	Sc11 % (normalized)	Sc11 # (normalized %)	TR % (normalized)	AB* % (normalized)	PAB % (normalized)	TR % (normalized)	Cl % (normalized)
1/2 Up	I^{st}	15	47 (100)	0 (0)	0.0 ± 0.0 (0)	87 (49)	0 (0)	67 (100)	87 (49)	47 (100)
	II^{nd}	27	63 (100)	0 (0)	0.0 ± 0.0 (0)	NF	0 (0)	67 (100)	NF	80 (95)
	III^{rd}	26	73 (100)	4 (4)	0.2 ± 0.8 (0)	0 (0)	NF	77 (100)	0 (0)	81 (95)
1/2 Lo		9	0 (0)	100 (100)	11.2 ± 0.6 (100)	89 (51)	89 (100)	0 (0)	89 (51)	0 (0)
	II^{nd}	19	0 (0)	100 (100)	11.0 ± 0.0 (100)	NF	89 (100)	0 (0)	NF	0 (0)
	III^{rd}	25	0 (0)	100 (96)	11.0 ± 0.0 (100)	80 (100)	NF	0 (0)	84 (100)	4 (5)
1/2 M	I^{st}	17	71 (100)	82 (54)	5.3 ± 1.3 (60)	100 (100)	82 (100)	0 (0)	89 (100)	6 (7)
	II^{nd}	26	73 (100)	77 (45)	4.7 ± 1.8 (37)	NF	41 (100)	0 (0)	NF	4 (5)
	III^{rd}	27	30 (100)	74 (49)	5.0 ± 3.5 (42)	52 (65)	NF	6 (10)	74 (69)	7 (7)
1/2 L	I^{st}	17	0 (0)	71 (46)	3.5 ± 2.1 (40)	0 (0)	0 (0)	53 (100)	0 (0)	82 (93)
	II^{nd}	16	0 (0)	94 (55)	8.1 ± 3.4 (63)	NF	0 (0)	50 (100)	NF	81 (95)
	III^{rd}	18	0 (0)	78 (51)	6.9 ± 1.7 (58)	28 (35)	NF	56 (90)	33 (31)	89 (93)

Fig. 8. Frequencies (%) of femur markers (Sc1, Sc11), tibia markers (TR, AB, PAB), tarsal markers TR and Cl differentiated from the four different halves from T1, T2, and T3 leg discs. Sc11 #: mean number of sensilla campaniformia differentiated. NF: not found. Parentheses: as in Fig. 6. For abbreviation see Figs. 2 and 3.

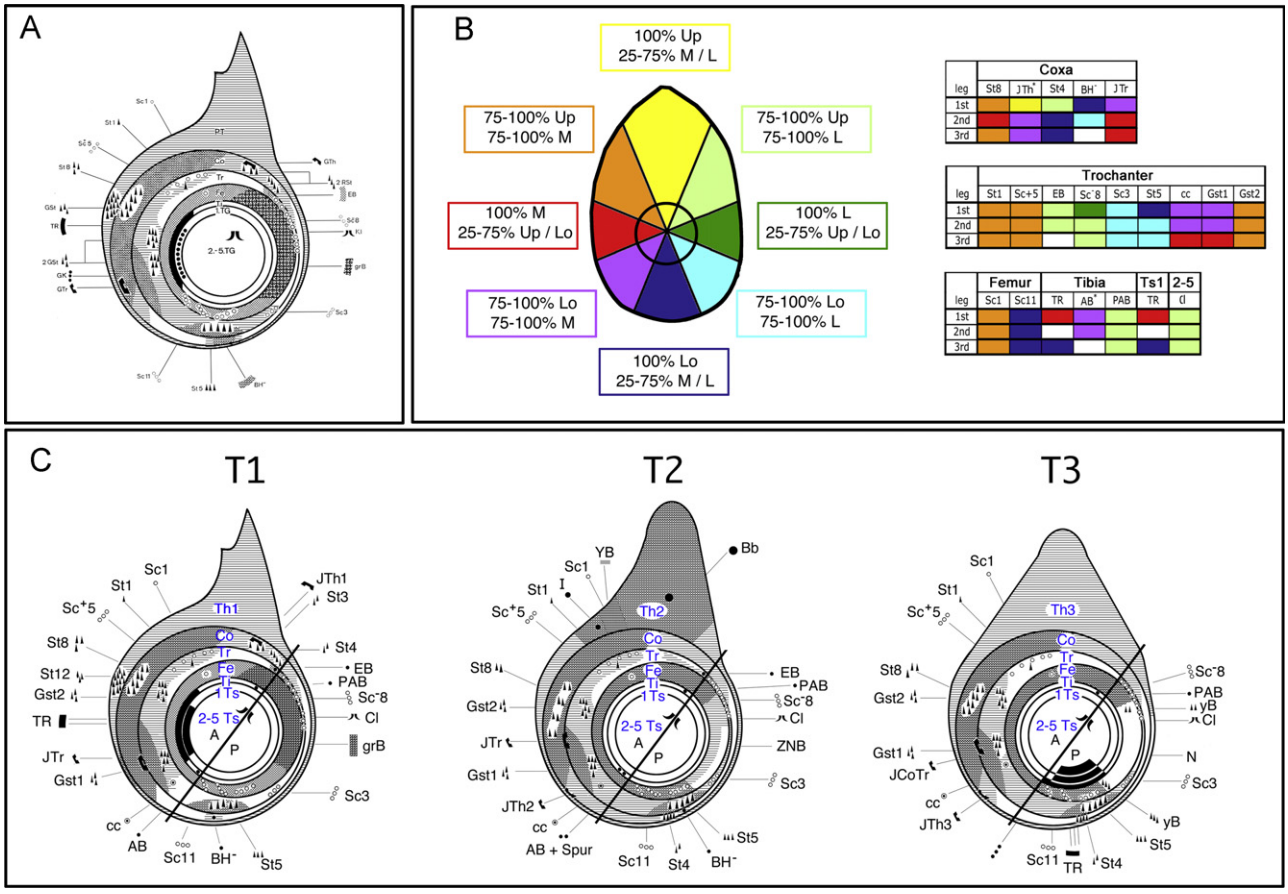


Fig. 9. Original (A) and revised (B, C) fate maps for T1–3 leg discs. (A) A previously published fate map of the T1 leg (Schubiger, 1968). Hair patterns are indicated by horizontal lines in thorax, coxa, and femur. Bristle primordia are indicated by black dots. Thus areas with bristles and hairs appear dark whereas hairy areas without bristles are gray. (B) Normalized frequencies (see Figs. 6–8) from two partially overlapping disc halves were used to plot each cuticular marker onto one of 8 sectors represented by eight different colors. If a marker differentiates with 100% frequency from one fragment and only 25–75% from the other fragment, it is designated to one of the four extreme sectors (yellow, dark green, dark blue, or red sector). For example, in all three discs, Sc11 is dark blue because it is found in 100% of 1/2 Lo fragments and between 25% and75% of 1/2 M and 1/2 L fragments. Structures are assigned to sectors orange, light green, light blue when two partially overlapping fragments produced this marker each with a very high frequency (75–100%). For example, in all three discs, St1 of the trochanter is formed by 100% of 1/2 Up and 100% of 1/2 M fragments, designating it to the mid-point of the orange sector. To place a marker to a region other than the mid-sector point, relative frequencies are also considered. For example, the primordium of Sc11 of all discs maps to the blue sector, but in T2 and T3 leg discs the Sc11 is shifted laterally within this sector because more Scs are formed by lateral halves (8.1 ± 3.4 for T2; 6.9 ± 1.7 for T3) than from T1 1/2 L fragments (3.5 ± 2.1) (C). For abbreviation see Figs. 2 and 3.

frequencies by the 1/2 M and 1/2 L fragments. Thus we have mapped it to the middle of the lower sector (Figs. 8 and 9B). We shifted this structure medially compared to the original fate map, because 1/2 M fragments produced on average 5.3 Sc (60%). No other adjustments from the original fate map were necessary (Fig. 9A, C [T1]).

Differences in the fate maps of T1, T2, and T3 leg discs

The different leg discs build different parts of the thorax. Based on clonal analysis, Steiner (1976) proposed that the sternum, the propleura, and epimerum I (Th 1) are formed from the T1 leg disc, while sternopleura and part of the hypopleura (Th 2) are formed by the T2 leg disc, and other parts of the hypopleura, episternum III, and epimerum III (Th 3) are formed by the T3 leg disc. With the exception of the sternopleura, these body parts lack good markers and produce rather similar hair patterns in the three legs. Thus a circumferential arrangement of the primordia in the discs can neither be rejected nor documented.

In control T2 legs, the sternopleura is a large structure and has 12 bristles (12.1 ± 2.3, $n=24$). We observed that all T2 leg disc fragments contributed to this body part, indicating a circumferential arrangement of its primordium. However, all 12 bristles were formed from the 1/2 Up fragment ($n=27$). We have placed the single bristle (I) in the medial and upper quadrant because only 1/2 Up and 1/2 M fragments differentiated this structure (1/2 Up, $n=27$; 1/2 M, $n=26$). The yellow bristles (YB) were also scored in the same fragments, thus we have placed their primordia in the same disc region (Fig. 9C [T2]). The big bristle (Bb) is formed from all 1/2 Up fragments ($n=27$). Furthermore, 1/2 M and 1/2 L fragments formed these structures with equal frequencies, justifying their position on the fate map (Fig. 9C [T2]). In control T2 legs, the big bristle(s) is (are) surrounded by about 8 bristles (Fig. 2A in situ, 4B disc transplantation control). We found these bristles in 70% of 1/2 M and 69% of 1/2 L fragments. Thus they were fate mapped to the upper part of the T2 leg (Fig. 9C [T2]).

The fate map location of the coxa/thorax joint differs between T1, T2, and T3 leg discs (Figs. 2, 3B, 6, 9C, J Th1, J Th2, and J Th3). The single joint of the T1 leg was easy to map (Fig. 9A; Schubiger, 1968) and its position is confirmed here (Figs. 6 and 9C [T1]). All 1/2 Up fragments of the T1 leg disc formed this joint. In addition, a majority of the 1/2 L fragments (65%) made this structure, thus its primordium has been placed slightly to the upper and lateral disc part (Figs. 6 and 9B, yellow sector and 9C [T1]). This joint was fate-mapped in T2 and T3 leg discs (J Th2, J Th3) at the opposite side compared to T1 leg discs since it was formed only by 1/2 Lo and 1/2 M disc halves (Figs. 6 and 9B, violet sector, 9C [T2, T3]).

As previously mentioned, T3 leg discs form two joints, but we were only able to map the one that was easy to identify by its big ball and clear socket structure. It has been put near the St4 of the coxa (Figs. 3, 6, and 9C [T3]). Other significant fate map differences were observed with the St3 and St4 markers of the coxa. St3 was only formed from T1 leg discs, and has been mapped to the same position in the fate map as J Th1 because these two structures were formed with similar frequencies by the different fragments (Figs. 6 and 9C [T1]).

Another interesting difference is the map position of St4 (coxa). In T1, St4 always formed from the 1/2 Up fragment, and more frequently from 1/2 L than from 1/2 M fragments (Fig. 6). Thus this marker was placed in the upper half of the disc slightly laterally (Fig. 9B, light green segment). In contrast, in T2 and T3, St4 was always produced by the 1/2 Lo fragment and with equal frequency by 1/2 M and 1/2 L fragments. Thus in T2 and T3 it was mapped to the medial-lateral border of the lower half of the leg discs (Figs. 6 and 9B, dark blue sector). We conclude that St4 maps almost opposite in T1 to where it maps in T2 and T3 leg discs.

Mapping TRs elicited another major difference. They are formed in T1 and T3 tibia and proximal tarsal segments (Fig. 8). All 1/2 M fragments of T1 leg discs formed TRs in the tibia and Ts 1 and they were formed with equal frequencies from 1/2 Up and 1/2 Lo fragments (Fig. 8). Therefore their primordia were mapped squarely in the medial half of the T1 disc (Fig. 9B red sector, and 9C [T1]). In T3 however, TR bristles were formed by all 1/2 Lo but only with modest frequencies from 1/2 M and 1/2 L fragments (65% 1/2 M; 35% 1/2 L, Fig. 8). Therefore TR bristles were mapped to the dark blue sector of the T3 disc (Fig. 9B).

Despite these different map positions of markers in T1, T2, and T3 leg discs all but two of the markers still mapped to the same compartment. The exceptions are the St 4 of the coxa and the TRs of tibia and tarsal segments found in T1 and T3 legs. In the T1 leg disc St4 of the coxa was mapped to the anterior compartment, but in the T2 and T3 leg discs this structure was mapped to the posterior compartment. In tibia and Ts 1 of the T1 leg disc TRs mapped to the anterior compartment but in the T3 leg disc these structures mapped to the posterior compartment. Therefore unexpected and significant differences in the patterning mechanisms exist for which there is no known cellular or molecular basis.

Minor differences in fate map positions

Two of the markers, $Sc^{-}8$ of the trochanter and Sc11 of the femur are spread out, and therefore occupy a rather large part of the cuticle. The $Sc^{-}8$ of the trochanter was found in all 1/2 L halves of the 3 different leg discs (100% normalized, Fig. 7). Comparing the numbers of Scs produced in 1/2 Up and 1/2 Lo fragments, we observed that 1/2 Lo fragments of the T1 leg disc formed 4.1 ± 0.4 Scs (46%), significantly higher than from 1/2 Lo fragments originating from T2 leg discs (0.9 ± 1.6; 13%) and from T3 leg discs (0.8 ± 1.6; 13%; Fig. 7). Thus in T1 leg discs we placed the $Sc^{-}8$ marker lower (dark green sector) than in T2 and T3 leg discs (light green; Fig. 9B, C). In the femur the Sc11 marker was observed only in 1/2 Lo halves (one exception) and with about equally high frequencies in 1/2 M and 1/2 L fragments from all leg discs justifying the map position to the dark blue sector (9B). However, more Scs were formed by T2 and T3 1/2 L fragments (8.1 ± 3.4; 63% T2; 6.9 ± 1.7; 58% T3) than from T1 leg discs (3.5 ± 2.1; 40%). This led us to place Sc11 of T2 and T3 leg discs to a slightly more lateral position than in T1 leg discs (Fig. 9C).

Our data has led us to map the BH^{-} bristle in the coxa more laterally in T2 compared to where it maps in T1 leg discs (Figs. 6; 9B dark blue vs. light blue sector, 9C). T3 leg discs do not form the BH^{-} bristle. Also, in the trochanter St5 maps more laterally in T2 and T3 compared to where it is in T1 leg discs (Figs. 7 and 9B dark blue vs. light blue, and 9C).

In T3, the trochanter structures cc and Gst1 differentiated from about a third of 1/2 Up fragments (33% cc, 40% Gst1; Fig. 7). Thus we placed their primordia in about the middle of the medial half of the T3 disc (Fig. 9B, red sector and 9C [T3]). In contrast, all but one of the 1/2 Lo fragments of T1 and T2 leg discs differentiated these structures (Fig. 7). Therefore we mapped cc and Gst lower in T1 and T2 compared to where they are in T3 leg discs (Fig. 9B violet sector and 9C).

The marker St8 of the coxa was formed by all three leg discs from all 1/2 M fragments (100%, Fig. 6). In T1 and T3, we observed that St8 usually differentiated from 1/2 Up fragments (Fig. 6), thus we mapped St8 to the upper-medial sector (Fig. 9B, orange sector; 9C [T1, T3]). But in T2 leg discs, we found that a significant fraction of 1/2 Lo fragments also formed this structure (Fig. 6), leading us to shift it towards the middle of the medial half (Fig. 9B, red sector). Moreover, T2 is distinct in that in a majority of cases, both 1/2 Lo and 1/2 Up fragments differentiated some sensilla trichodea (Fig. 6). We interpret this to mean that in T2 leg discs, the St8 sensilla are spaced further apart on the fate map than they are in the other leg discs (Fig. 2A, St8; Fig. 3B1, St8; Figs. 6 and 9C).

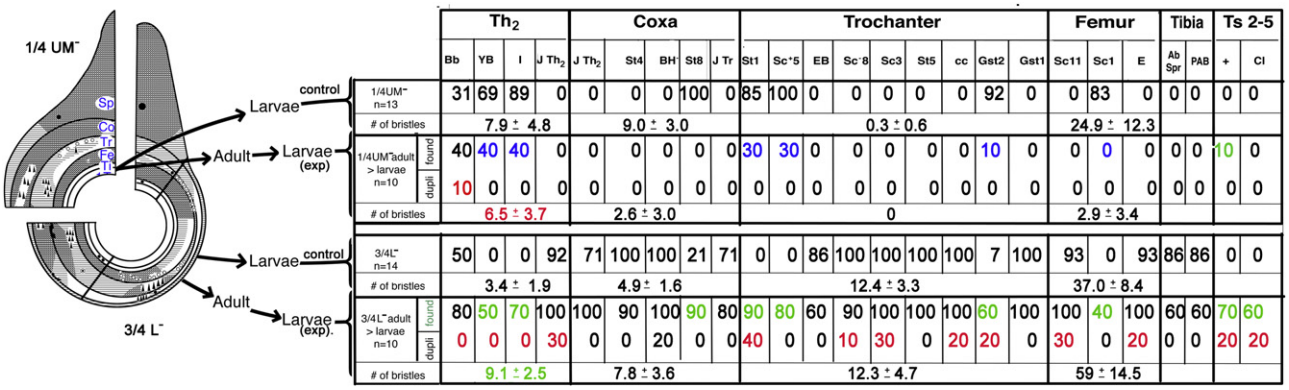
No differences in the fate maps

$Sc^{+}5$, a trochanter marker was formed from all 1/2 Up and 1/2 M fragments of all leg discs (Figs. 5B and 7, 100%, 1/2 Up; 100%, 1/2 M) but never from 1/2 Lo and 1/2 L fragments. This allowed us to map the $Sc^{+}5$ marker onto the fate map to the upper-medial quadrant for all discs (Fig. 9B, orange; 9C). Similarly St1, Gst2 (trochanter) and Sc1 (femur) showed the same distribution pattern in all discs, so that they also mapped to this sector (Fig. 9B, orange; 9C). This map position is supported by our observation that the 1/4 UM^{-} fragments also differentiated them (Schubiger, 1971; and Fig. 10). EB (which does not appear in T3 legs), PAB, and the claw of all three legs mapped to the middle of the upper-lateral quadrant of the T1, T2 and T3 leg discs (Figs. 8 and 9B, light green; 9C). Likewise we mapped Sc3 to the same lower-lateral quadrant in all three discs (Figs. 5B; 7; 9B light blue; 9C). In summary St1, $Sc^{+}5$, Sc 3, Gst2, EB of the trochanter, Sc1 of the femur, PAB of the tibia, and the Cl all mapped to the same disc location in all three leg discs (Fig. 9B and C).

Developmental plasticity of T2 and T3 leg discs

When T1 leg disc fragments, containing cells of the anterior compartment are allowed to proliferate prior to differentiation, they will regenerate missing structures. During regeneration, some cells undergo major fate changes, cross compartmental boundaries and even take on the identity of other discs, and make, for example, wing structures. This phenomenon is known as transdetermination (TD) (Hadorn, 1965, 1978). Interestingly, Steiner et al. (1981) reported that, in stark contrast to T1 leg discs, fragments from T2 and T3 leg discs only very rarely transdetermine to wing (59% for T1 leg disc vs. 4% each for T2 and T3 leg discs). They also have reported that in these experiments, T1 and T2 leg discs overgrew to the same degree. This is unexpected because TD frequency usually correlates with overgrowth (Tobler, 1966). Thus the failure of TD in T2 leg discs is not explained by the lack of overgrowth. So we asked what structures are made from the outgrowth in T2 and T3 leg disc fragments.

We chose to test such developmental plasticity using the 3/4 L^{-} fragment (Fig. 10), because in T1 leg discs, the capacity of this fragment is well characterized: it duplicates fate map structures, regenerates missing patterns, and transdetermines to wing with a



		Th2				Coxa					Trochanter								Femur			Tibia		Ts 2-5	
		Bb	YB	I	J Th2	J Th2	St4	BH	St8	J Tr	St1	Sc+5	EB	Sc+8	Sc3	St5	cc	Gst2	Gst1	Sc11	Sc1	E	Ab Spr	PAB	+	Cl
1/4UM⁻ n=13		31	69	89	0	0	0	0	100	0	85	100	0	0	0	0	0	92	0	0	83	0	0	0	0	0
# of bristles		7.9 ± 4.8				9.0 ± 3.0					0.3 ± 0.6									24.9 ± 12.3						
1/4UM⁻adult > larvae n=10	found	40	40	40	0	0	0	0	0	0	30	30	0	0	0	0	0	10	0	0	0	0	0	0	10	0
	dupli	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
# of bristles		6.5 ± 3.7				2.6 ± 3.0					0									2.9 ± 3.4						
3/4L⁻ n=14		50	0	0	92	71	100	100	21	71	0	0	86	100	100	100	100	7	100	93	0	93	86	86	0	0
# of bristles		3.4 ± 1.9				4.9 ± 1.6					12.4 ± 3.3									37.0 ± 8.4						
3/4L⁻adult > larvae n=10	found	80	50	70	100	100	90	100	90	80	90	80	60	90	100	100	100	60	100	100	40	100	60	60	70	60
	dupli	0	0	0	30	0	0	20	0	0	40	0	0	10	30	0	20	20	0	30	0	20	0	0	20	20
# of bristles		9.1 ± 2.5				7.8 ± 3.6					12.3 ± 4.7									59 ± 14.5						

Fig. 10. Testing developmental plasticity of T2 leg disc fragments (1/4 UM⁻ and 3/4 L⁻ fragments). Control fragments were injected into late wandering larval hosts. To test for regeneration (experiment [exp]) the same types of fragments were injected into adult females and, after 7 days, transplanted into metamorphosing larvae. The frequencies of regenerated structures are indicated in green; duplicated markers in red; loss of fate map structures in blue. For abbreviations see Figs. 2 and 3.

high frequency (59%; Schubiger, 1971). In the following experiments we removed the primordia for tarsal segments 2–5(Fig. 10) to test for the capacity to regenerate missing tarsal structures. For controls, we determined the fate map structures of the 3/4 L⁻ fragment and its complement, the 1/4 UM⁻ piece, by injecting them into late third instar larvae. We observed that the 1/4 UM⁻ fragment, but not the 3/4 L⁻ fragment, gave rise to the YB (sternopleura), St1, Sc⁺5 (trochanter), and Sc1 (femur), and are thus the best markers to test for regeneration (Figs. 6 and 7). For the same reason the I-bristle (Sp) and the St8 (coxa) also served as good regeneration markers (Fig. 10). For testing distal regeneration, the differentiation of an additional tarsal segment and the claw organ was scored.

In the experimental series, T2 leg disc fragments were retrieved from the host after 7days of in vivo culture, and injected into larval hosts to allow fragments to differentiate adult structures. During in vivo culture the 1/4 UM⁻ fragments did not grow very much and we did not find any cases where the fragments had produced structures from the 3/4 L⁻ fate map (Fig. 10). In one case out of ten, the 1/4 UM⁻ fragment seemed to have regenerated some tarsal bristles, but were difficult to assign to a specific tarsal segment. We conclude that, in contrast to the T1 disc, the T2 1/4 UM⁻ leg disc fragment has no or very limited ability to regenerate.

Comparing 1/4 UM⁻ experimental fragments with 1/4 UM⁻ controls, we observed fewer markers after in vivo culture. For example, in controls 85% of the 1/4 UM⁻ fragments formed the marker St1 of the trochanter, but after in vivo culture and differentiation this structure was only observed in 30% of cases (Fig. 10). One explanation for this is that cells in smaller fragments generally survive poorly. However, several observations argue against this idea. First, the same 1/4 UM⁻ fragment from T1 leg discs can regenerate an entire leg (Schubiger, 1971). Thus fragment size is not limiting regeneration capabilities. Furthermore, we observed that the cell loss was not uniform. The loss of distal structures was very high, and the bristle number loss in coxa was significant but not as severe as observed in the femur. Meanwhile, in Th2 the loss of bristles was not statistically significant (7.9 ± 4.8 Sternopleura bristles in larval controls vs. 6.5 ± 3.7 bristles in the regeneration experiment). In one case we even found that Sp bristles had duplicated and in another case we observed clear duplications of the big bristle (Bb) of the Sp (Fig. 10).

Interestingly however, when we compared the structures generated from the 3/4 L⁻ larval disc control with the experimental set, we found many cases of T2 regeneration. The clearest evidence was the regeneration of markers such as YB (Sp), St1, Sc⁺5 (trochanter), Sc1 (femur), and tarsal segments 2–5 which were not present in the 3/4 L⁻ controls, but were formed by the complementary 1/4 UM⁻ control (Fig. 10). Most impressive were two experimental cases that had formed all structures and bristles in numbers not different from whole T2 leg disc transplantation controls (data not shown). In two other cases the regenerated tarsal segments were also duplicated (Figs. 10 and 11). Interestingly, 3/4 L⁻ pieces from T2 leg discs regenerated missing patterns more frequently than they duplicated pattern elements of the fate map (Fig. 10). With T1 leg discs, we reported the opposite, namely that the frequency of pattern duplication in 3/4 L⁻ fragments dominates that of regeneration (Schubiger, 1971).

We carried out the same experiments (3/4 L⁻ and 1/4 UM⁻ fragments) with T3 leg disc fragments. Again, we performed controls by injecting the complementary fragments into late wandering larval hosts. As with T2 leg discs, the 1/4 UM⁻ fragment ($n=6$) produced fewer structures after in vivo culture and differentiation than the control ($n=12$). The 3/4 L⁻ fragment of T3 leg discs regenerated the missing tarsal segments in 7 out of 11 cases. Regeneration of proximal structures was observed in 9 out of 11, and in 4 of them, both regeneration and duplication had occurred. We found one case of possible leg to wing TD. We conclude that all three leg discs regenerate missing distal primordia, but missing proximal markers are regenerated more frequently with T2 and T3 leg disc fragments than with T1 fragments.

Discussion

The progenitors of the leg discs are determined first in the embryo as a population of 15–20 cells, with two clusters in each of the three thoracic segments (Cohen, 1993). Discs proliferate during larval life and cells become restricted to anterior or posterior compartments, as well as to their proximal/distal position (Steiner, 1976; Estella et al., 2012). During metamorphosis, leg discs evaginate by cell rearrangement and breaking of the peripodial epithelium (Bosch et al., 2005). Big cell shape changes and some proliferation also occur during this period (Graves and Schubiger, 1982). Pupal cuticle apolysis occurs at about 18 h after puparium formation (APF) and the everted leg primordium begins to constrict, mainly circumferentially up until 36 h APF (Fristrom and Fristrom, 1993).

Conservation of cuticular markers across Drosophila species

We were interested to see whether the cuticular markers and pattern constancy and differences described here for the three

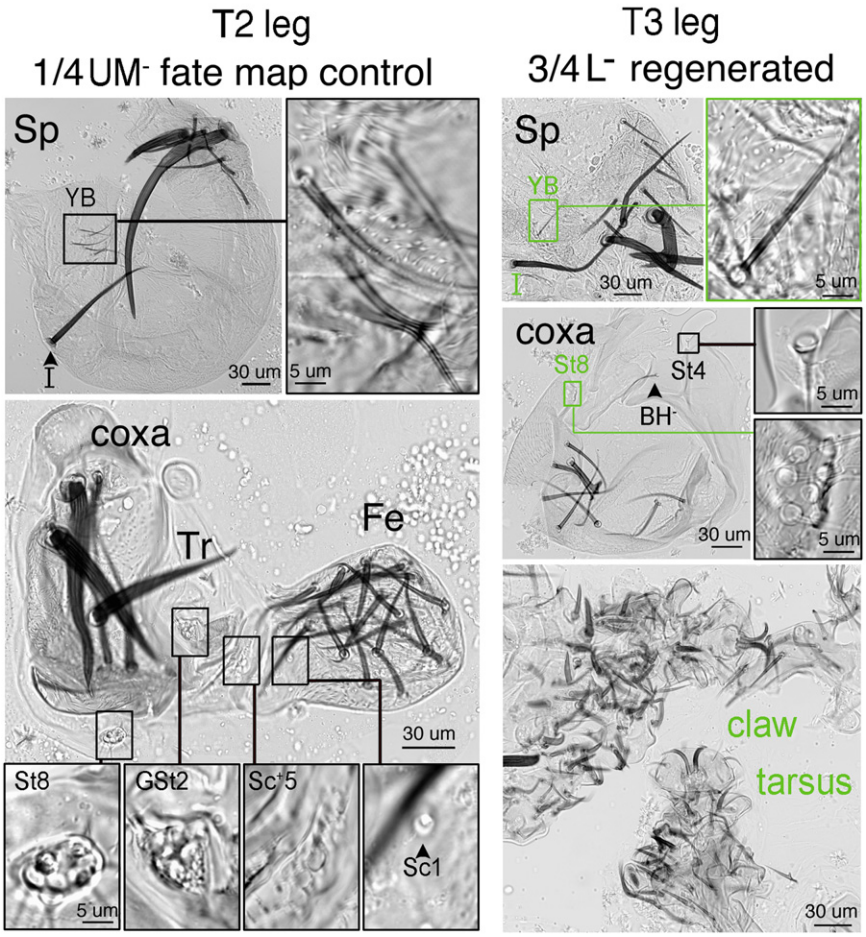


Fig. 11. Samples of regenerated proximal (YB, St8) and distal markers (claw, several tarsal segments) that also duplicated (for abbreviations, see Figs. 2 and 3).

pairs of legs of *D. melanogaster* are also observed in legs across *Drosophila* species. Previously, a short descriptive study of sensory fields of four *Drosophila* species (*D. melanogaster*, *D. virilis* and the two Hawaiian species *D. planitiobia* and *D. disjuncta*) reported no differences of the sensory fields and their positions among the different legs (Hoikkala and Moro, 2000). We decided to have another look at this because of the specific differences we found between the different legs both in terms of sensory fields and bristle patterns (Figs. 2 and 3). Earlier comparative studies of different *Drosophila* species either concentrated on bristle patterns but not bristle number of the three leg pairs (Hannah-Alava, 1958; Hollingsworth, 1964), or on bristle patterns of only one leg segment (e.g. femur; Stern, 1998). Therefore we analyzed the three leg pairs of *D. hydei, D. virilis, D. bifurca*, and *D. nanoptera* and compared them to *D. melanogaster*.

First we asked whether there were differences in bristle number between species. Overall, we found that *D. hydei, D. virilis,* and *D. bifurca*, but not *D. nanoptera*, have significantly more bristles in the coxa, trochanter, and femur ranging from 30% to 100% more bristles. We did not find TRs in T2 legs in any of the *Drosophila* species. However, TRs were always observed in T1 and T3 legs. *D. melanogaster* has 5 or 6 TRs in the tibia of T1 legs, while the bigger species like *D. hydei* and *D. bifurca* have 9 TRs respectively between 9 and 11 TRs. In contrast, the number of TRs in the T3 leg did not differ between the five species. TRs were also observed in Ts 1 of T1 and T3 legs. In leg T1 of the five *Drosophila* species there are 10 or 11 TRs. In leg T3 there are 13 or 14 TRs. Other differences between the three leg pairs are conserved among all five species. For example, all five species have two rows of Sts (St3 and St4) in the coxa of T1 leg, but only one row in T2 and T3 legs. As in *D. melanogaster* the BH bristle of the coxa and the EB bristle in the trochanter are missing in T3 legs (Figs. 2C, 3C, and 12A, B). Occasionally the trochanter of T2 legs in *D. virilis* has 2 EBs (Fig. 12C). Thus we conclude that there are significant pattern differences between the three legs that are conserved between species, and are regulated by *Scr* and *Ubx*.

Other markers, including the number of sensilla, are remarkably conserved among the five *Drosophila* species. Likewise, no differences were observed in the number of sensilla trichodea and campaniformia in sensory fields of the three different leg pairs of the five species (data not shown). For example in the coxa, the eight Sts of the St8 marker are arranged in two rows of four sensilla (Fig. 2A). This same arrangement was observed in the coxae of all three pairs of legs from the tested *Drosophila* species. The same conservation of markers was observed in the trochanter sensory fields (subset of markers): St1, $Sc^{+}5$, $Sc^{-}8$, Sc3, St 5, Gst1, and Gst2 (Fig. 12). Also the arrangement and number of Sc11 into three rows of 3, 4, and 4 sensilla in the femur and the position of Sc1 (Fig. 12) are the same in all species analyzed even though *D. hydei* is much larger than *D. melanogaster*. We conclude that the number of sensory organs (the markers), and the number of sensilla within the marker is evolutionary conserved despite the different size of the flies. In contrast, bristle number varies and correlates with leg segment length and size of the fly. Hence, the number of bristles is likely to be a function of primordium size, while the number of sensory organs is size independent. Frantsevich and Gladun (2002) analyzed the number of sensilla in the different sensory fields of 20 cyclorrhaphan flies, some big,

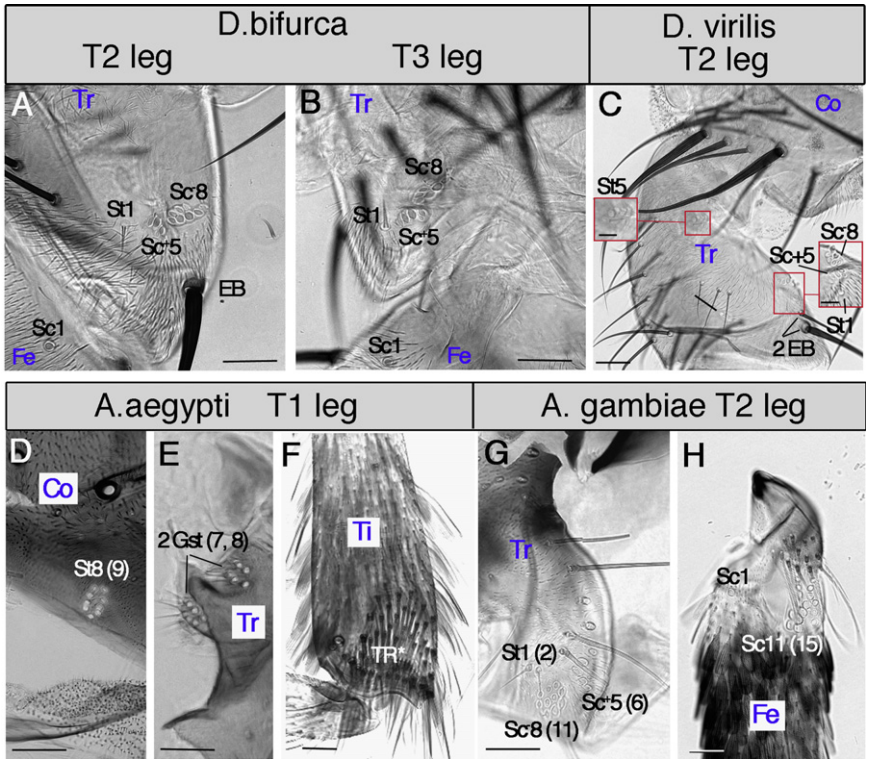


Fig. 12. Comparison of sensory fields in different *Drosophila* species (A–C) and different mosquitoes (Nematocera; *Aedes (A.) aegypti*; (D–F) and *Anopheles (A.) gambiae* (G, H)). Markers that do not differ between the three pairs of legs in *D. melanogaster* such as St1, $Sc^{+}5$, $Sc^{-}8$ in the trochanter (Tr) and Sc1 in the femur (Fe) also do not differ in *D. bifurca* (A, B) and *D. virilis* (C). Those that differ in *D. melanogaster* differ in other *Drosophilia* species as well (no EB in T3 leg; B). In leg T2 we occasionally found 2 EBs in *D. virilis* and in other large *Drosophilidae* (C). In *A. aegypti* (D–F) and *A. gambiae* (G, H) we found the same sensory fields in the corresponding place, but specific fields have more sensilla. For example, the St8 sensory fields of the *Drosophilidae* coxae always have 8 Sts but in *A. aegypti* we found 9 [D, St8 (9)]. A similar increase was observed in sensory fields that vary in *Drosophila*. For example, the 2 GSts of the trochanter, the *Drosophilidae* have 6 or 7 Sts, *A. aegpyti* has 7, 8, or 9 Sts (E). The $St^{+}5$ in *Drosophila* has between 4 and 7 Scs, in *A. gambia*, between 6 and 9 Scs (G, $Sc^{+}5$ (6)) and the St1 of the trochanter has 2 Sts (G, St1 (2)). $Sc^{-}8$ in *Drosphilia* has 6 to 9 Scs. In *A. gambiae* we found between 8 and 12 Scs (G, $Sc^{-}8$ (G, 11)). In the femur the Scs in Sc11 are increased to 15 but are still arranged in three rows (H, Sc11 (15)). As in all tested *Dosophilidae*, the tibia of leg T1 and T3 of *A. gambiae* have TRs but here they are arranged in a triangular shape that is called tibial scraper TR* (F).

some small. The study involved only coxa and trochanter and only T2 legs. They found that the number of sensilla in a specific sensory field is the same, except in ectoparasites and bot flies. However the larger cyclorrhaphan flies have larger sensilla.

Genetic pathways and fate-map markers

The three pairs of legs are patterned by the same mechanisms (Brook et al., 1996; Cohen, 1993; Held, 1995), and their segmental characteristics are defined by the expression of the Hox genes *Scr* in T1 (Struhl, 1982) and *Ubx* in T3 (Casanova et al., 1985; Kerridge and Morata, 1982; Lawrence et al., 1979; Rozowski and Akam, 2002; Stern, 1998).

The cuticular markers we have used to generate fate maps are composed of sensory structures. Many of them (e.g., St8 in the Coxa, St5, $Sc^{+}5$, $Sc^{-}8$, and the 2 GSt's in the trochanter and Sc11 and Sc1 in the femur) are identical in all three pairs of legs and do not differ between the sexes. Therefore the homeotic genes *Scr* and *Ubx* are not directly responsible for defining these markers. Rather, the development and differentiation of sensory structures are regulated by the three proneural genes: *achaete (ac), scute (sc),* and *lethal of scute (l'sc)*. They define the neural precursors, which then express *asense (asc)* to induce sensory organ development (Negre and Simpson, 2009). In addition to the three proneural genes, all *Drosophilidae* have an additional gene, *pcl*, within the *ac-sc* gene complex (AS-C). Interestingly, different species of the *Drosophilidae* have different numbers of *pcl* genes; *D. virilis* has three and *D. melanogaster* has only one *pcl* gene (Negre and Simpson, 2009). While the functional importance of *pcl* gene rearrangements has not been assessed, we found no difference between the two species either in terms of number of markers and numbers of sensilla within the markers. This indicates that changing the landscape of *pcl* gene number does not change the sensilla pattern.

Negre and Simpson (2009) have proposed a model for AS-C gene duplications in insect evolution. All insects have one *asc* gene but the numbers of the other three proneural genes vary (*ac-sc* homologous genes are known as *ASH* in other insects). We asked whether these gene duplications are correlated with the number of the fate map markers and/or their number of sensory sensilla within the markers. Dipterans are subdivided into *Brachycera* (e.g., *Drosophilia*) and *Nematocera* (e.g., mosquitoes such as *Anopheles, Culex*, and *Aedes*). *Anopheles* does not have a duplication of the *ASH* gene, whereas *Culex* and *Aedes*, which are on a different branch of the evolutionary tree, have one duplication (Negre and Simpson, 2009, their Fig. 3). The four species all have identical numbers of sensilla in the following sensory fields: in coxa of T1 legs, two rows of Sts (St3 and St4); in T2 and T3 legs, one row of Sts (St4); in the trochanter, St5; and in the femur, Sc1 (Spinner, 1969, *Culex* T1 leg; Frantsevich and Gladun, 2002; unpub. obs). Thus this precise repetition of pattern is also independent of *ASH* gene copy number.

With respect to the other sensory fields we and others observed small but consistent pattern deviations from the *Drosophilidae*. For example, in the coxa of all three leg pairs of *Anopheles gambiae*, *Culex pipens* (T1 leg only; Spinner, 1969), and *Aedes aegypti*, the St8 has one additional sensillum trichodeum but, as in *Drosophila*, is still arranged in two rows (Fig. 12D). An increase of

just one sensillum is also observed in other markers that show no or little variation in their sensilla numbers in the *Drosophilidae,* such as the Sc3, St1 of the trochanter (T1 leg; Spinner, 1969 and Fig. 12G). In the femura of these dipterans Sc11 is still arranged in three rows but there are 3 or 4 more Scs in the mosquitoes (Spinner, 1969; Frantsevich and Gladun, 2002; Fig. 12H). Sensory fields that vary in their sensilla numbers in *Drosophila*, such as GSt1, Gst2, $Sc^{+}5$, and $Sc^{-}8$ also vary in mosquitoes and have an increase in sensilla numbers (T1, Spinner, 1969, and Fig. 12E). Comparing sensory fields not just by numbers but also by shape we observe that $Sc^{+}5$ and $Sc^{-}8$ differ more than all the others (Figs. 2, 3 and 12A, G). Therefore, as we have stated above, we find that there is no correlation between the numbers of *ASH* genes and the patterning of sensory fields.

Looking at the distribution of TRs in the different pairs of legs in *Drosophila, Anopheles*, and *Aedes* we found TRs in T1 and T3 legs, but never in T2 legs. *Anopheles* and *Aedes* have no TRs in tarsal segments and only one in the Tibia of T1 and T3 legs (this "row" was originally described as a "tibial scraper"; Marshall, 1966; and Fig. 12F).

In general, it is thought that leg patterning mechanisms are conserved between holometabolous insects. Expression of leg patterning genes such as *en*, which specifies the anterior–posterior axis, *wg* and *dpp*, which regulate dorsal and ventral axis and *exd/hth, dac*, and *Dll* which control proximal–distal axis are conserved between *Drosophila* and the beetle *Tribolium castaneum* (Williams, 1999; Nagy and Carroll, 1994; Sanchez-Salazar et al., 1996; and reviewed in Rozowski, 2002). The expression of these genes does not differ between the three pairs of legs. Here, for fate mapping leg discs, we have used sensory fields that also do not differ between species. This made us wonder how these sensory fields are evolutionary conserved in flies (Dipterans). Frantsevich and Gladun (2002) studied coxa and trochanter in T2 leg of 205 species from 68 fly families. They observed the same number of sensory fields (9) in all of them as we describe here for *Drosophila melanogaster* (Coxa: St8 and St4; Trochanter: 2 Gst's, St5, St1, $Sc^{-}8$, $Sc^{+}5$, and Sc3). In Cyclorrhapha (*Drosophila, Musca*, etc.) the authors reported that the "number of sensilla is standard", as it is in *Drosophila*, and independent of body size. In nematocerans and noncyclorrhapan brachchycerans (e.g., *Chironomidae*) the same sensory fields are observed but they contain more sensilla. Thus we speculate that the generation of these sensory fields must be more robust than the positioning of the bristle patterns, which differ more between species and between the different pairs of legs. In addition some of the dipterans, for example *Aedes*, carry a dense pattern of scales, another sensory structure, but maintain the same sensory fields. Is it then possible that *wg* and *dpp* signaling directly regulate the specific position of sensory fields? This would be in contrast to bristle patterning where *hairy* is expressed in stripes in femur and tibia and delimits *ac/sc* expression which in turn defines bristle rows in *Drosophila* legs (Orenic et al., 1993).

Homologous fragments of different leg discs differ in their developmental plasticity.

In one of our earlier publications we reported that 3/4 L fragments of the T2 leg disc frequently regenerate after in vivo culture, whereas the homologous fragment from the T1 leg discs duplicated in most cases and only fragments from the T1 leg discs transdetermined. We repeated this experiment and made the same observations. In addition we tested the developmental plasticity of the complementary fragment, the 1/4 UM piece. We performed the same experiment with the T3 leg disc and found that these fragments behave like those from T2 leg discs. Thus the T1 leg disc has more developmental plasticity than the T2 or T3 leg discs. We think that increased plasticity may occur because disc fragmentation cuts through two cell layers, the peripodial epithelium and the disc proper. A vertical cut runs through *en*-expressing cells in all leg discs. But only the peripodial cells in T1 leg discs express *hh*. During wound healing, peripodial cells at the cut fuse transiently with anterior columnar cells and provide them with Hh, which then down regulates Ci and activates *en* (Gibson and Schubiger, 1999; Schubiger et al., 2010).

There are at least two other differences that have to be mentioned: Steiner (1976) observed that in T1 leg discs, *Minute* cell clones induced up until the mid-first instar overlap between left and right legs. Such overlaps have never been observed in T2 and T3 legs. This indicates that hemisegment identity in the T1 segment is established after it has already occurred in T2 and T3 leg discs. Only the pair of T1 leg discs shares a common peripodial epithelium. This difference might underlie the differences in developmental plasticity necessary for regeneration (Gibson and Schubiger, 1999; Gibson et al., 2002).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.05.025.

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